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Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment

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Abstract

We investigated in the black-headed gull whether female deposition of antioxidants and immunoglobulins (enhancing early immune function), and testosterone (suppressing immune function and increasing early competitive skills) correlate suggesting that evolution has favoured the mutual adjustment of different pathways for maternal effects. We also took egg mass, the position of the egg in the laying sequence and offspring sex into account, as these affect offspring survival. Yolk antioxidant and immunoglobulin concentrations decreased across the laying order, while yolk testosterone concentrations increased. This may substantially handicap the immune defence of last-hatched chicks. The decrease in antioxidant levels was greater when mothers had a low body mass and when the increase in testosterone concentrations was relatively large. This suggests that female black-headed gulls are constrained in the deposition of antioxidants in last-laid eggs and compensate for this by enhanced testosterone deposition. The latter may be adaptive since it re-allocates the chick's investment from costly immune function to growth and competitive skills, necessary to overcome the consequences of hatching late from an egg of reduced quality.

Introduction

Parents influence the fitness of their offspring by transferring resources to them in addition to their genome. The advantage of such parental or maternal effects (Mousseau & Fox, 1998) is that they can be adjusted to the prevailing post-hatching conditions, since the effects may arise from the environment as the mother experiences it. One may expect that evolution has favoured the adjustment of different maternal resources towards each other to maximize maternal fitness. Birds are excellent models for studying maternal effects, producing relatively large eggs that represent a substantial maternal investment that markedly influences post-hatching development and survival (reviewed by Williams, 1994; Christians, 2002). This maternal resource allocation takes place in a short time window, and no further adjustments of the egg components are possible once the egg is laid.

One important component of bird eggs that is present in substantial levels is maternally derived yolk androgens. Experimentally increased levels of maternal yolk androgens can influence hatching time (Eising *et al.*, 2001; Sockman & Schwabl, 2000) and enhance begging behaviour and postnatal growth (Schwabl, 1993, 1996; Eising *et al.*, 2001; Eising & Groothuis, 2003), the first two probably by strengthening of the neck muscle (Lipar & Ketterson, 2000). In this way, the enhanced levels of maternal hormones in last-laid eggs of natural clutches of many bird species may mitigate the negative consequences of hatching asynchrony for the last-hatched chick (Schwabl, 1993; Eising *et al.*, 2001; reviewed in Groothuis *et al.*, 2005b). On the other hand maternal

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yolk androgens may entail costs for the offspring. In several bird species testosterone suppresses immune function (Ketterson & Nolan, 1999; Duffy *et al.*, 2000; Peters, 2000, but see Ros *et al.*, 1997; Hasselquist *et al.*, 1999), as does experimental elevation of yolk androgens in chicks (Hirota *et al.*, 1976; Groothuis *et al.*, 2005a; Müller *et al.*, 2005). Experimental elevation of yolk androgens is widely recognized not only to suppress antibody responses via premature regression of the bursa of Fabricius (Hirota *et al.*, 1976) but can also enhance the T-lymphocyte compartment by promoting thymic hyperplasia (Marsh, 1992). The finding that Great Tit (*Parus major*) mothers lower androgen deposition in their eggs when breeding in nest boxes containing a high load of parasites is in line with the immunosuppressive effect of yolk androgens (Tschirren *et al.*, 2004). In addition, androgens may promote oxidative stress resulting from accelerated growth (von Schantz *et al.*, 1999).

The effect of maternally derived androgens on immunity may indeed be of great importance for a young chick. At hatching the chick leaves the sealed environment of the egg and is confronted with a spectrum of infectious agents that can cause morbidity and mortality while their immune system is not yet fully developed (Apanius, 1998). Therefore, it may be adaptive for avian mothers to provide the egg, and thus enhance the immune defence of their offspring, with maternal immunoglobulin G (IgG) (Gasparini *et al.*, 2001; Buechler *et al.*, 2002; Saino *et al.*, 2003) and antioxidants including carotenoids and vitamin E (Royle *et al.*, 2001; Blount *et al.*, 2002; Saino *et al.*, 2003). IgG, deposited in the egg yolk, provides protection during the vulnerable period between hatching and maturation of endogenous immune function (reviewed by Grindstaff *et al.*, 2003). The protective role of maternally derived immunoglobulins has, for example, been demonstrated by experimental infections of domestic birds (Kariyawasam *et al.*, 2004). Furthermore, transmission of maternal immunoglobulins has been documented in a variety of wild bird species and is positively correlated with nidicolous ectoparasite levels (Gasparini *et al.*, 2001; Buechler *et al.*, 2002). In domestic birds, the ability of the chick to raise an antibody-mediated immune response develops during the first and second week post-hatch (Apanius, 1998). If yolk androgens suppress neonatal antibody responses, then evolution may have favoured mothers that compensate for this potential cost by transferring an increased quantity of their preformed immunoglobulins into the egg to promote their fitness.

Maternally transmitted antioxidants (particularly carotenoids and vitamin E) are critical for embryonic development as they protect growing tissues from oxidative damage including lipid peroxidation (Surai *et al.*, 1999; Surai, 2002). Pertinent to this study, antioxidants are widely believed to enhance immune function. For example, carotenoids have been shown to enhance T-cell-mediated immunity early post-hatching in barn swallow chicks *Hirundo rustica* (Saino *et al.*, 2003, for similar effect in adult zebra finches *Taeniopygia guttata* see Blount *et al.*, 2003; McGraw & Ardia, 2003). Maternal transfer of antioxidants to the yolk may also entail a cost for the mother. Antioxidants can only be obtained in the mother's diet and their availability may thus be suboptimal (reviewed by Brush, 1990), while deposition into the yolk probably reduces their availability for the maternal self-maintenance. Indeed recent work on lesser black-backed gulls *Larus fuscus* has shown that egg-laying capacity can be constrained by carotenoid availability (Blount *et al.*, 2004).

To understand the patterns of deposition of these egg components, egg size or mass, which is related to the amount of nutrients, should also be taken into account. Egg nutrients determine energetic resources for growth as well as the development and expression of the immune system, both of which are energetically costly (reviewed by Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000).

We may expect that evolution has favoured the deposition of immunosuppressive androgens, and immuno-enhancing IgG, antioxidants and nutrients to be adjusted in relation to each other to increase maternal fitness. They should also be adjusted to the need to mitigate hatching asynchrony and the risk of infectious diseases. The expectation that mothers compensate for immunosuppressing effects of elevated androgens in their last eggs by transferring more IgG and antioxidants to these eggs has, however, not been supported by previous studies of the lesser black-backed gull. Levels of androgens increased while those of carotenoids, vitamin E and maternal IgG decreased with laying order (Royle *et al.*, 2001; Blount *et al.*, 2002). This suggests a negative association between immunosuppressing and immuno-enhancing factors. However, there are several reasons to investigate interactions among such maternal effects in more detail. First, the findings were obtained with averaged values over eggs of clutches of many different females, despite substantial variation among these eggs and among clutches in the levels of these yolk compounds. This opens the possibility that, within these trends at the population level, individual females may still adjust to some extent the transfer of one compound to another, either at the level of the total clutch or in the rate of increase or decrease across the laying sequence. For example, we expect that a stronger decrease of carotenoids or antibodies over the laying order should correlate with a less strong increase in androgen levels to minimize detrimental effects on the chick's immunity.

In addition, the (adjustment of) deposition of egg components may depend on the sex of the offspring. In many avian species, early nestling mortality differs between male and female offspring. In gulls, the male embryo is more vulnerable to low egg quality (Nager *et al.*, 1999, 2000), and evolutionary theory predicts that

males may, therefore, require a relatively pronounced maternal adjustment of levels of egg components to each other. Finally, the deposition patterns of yolk compounds may be related to the body condition of the female.

We measure within- and between-clutch variation in egg mass, yolk testosterone, IgG, carotenoids and vitamin E, as well as the relations between these variables and with position of the egg in the laying order, offspring sex and maternal body mass in the black-headed gull *Larus ridibundus*. This is an appropriate model organism, producing eggs that contain high levels of maternal androgens which vary systematically between and within clutches (Eising *et al*., 2001; Groothuis & Schwabl, 2002), and their functional consequences for growth and behaviour have been convincingly demonstrated (e.g. Eising *et al*., 2001; Eising & Groothuis, 2003). In this species immune-relevant egg components such as IgG and antioxidants may be particularly important as it breeds in extremely dense colonies in which the aggregation of large numbers of birds during the breeding season enhances the risk of infectious diseases (Brown & Brown, 1986; Loye & Zuk, 1991; Tella, 2002). Indeed, concentrations of maternal IgG in the yolk are positively related to breeding density and presumably to the potential risk of infection (Müller *et al*., 2004b). Finally, early nestling mortality in this species is related to hatching asynchrony, T-cell-mediated immunity and sex of the offspring (Müller *et al*., 2003).

Material and methods

Study species and data collection

Black-headed gulls are monogamous, colonial breeders. The clutch typically consists of three eggs, which are laid over a 3 to 5-day period (Cramp & Simmons, 1983). In 2001, nests of several neighbouring black-headed gull subcolonies (300–1000 breeding pairs) along the north-east coast of the Netherlands were checked once a day for egg laying. Freshly laid eggs were marked with nontoxic ink referring to the position within the laying order and laying date. We collected 20 complete clutches on the day of clutch completion. The eggs were weighed to the nearest 0.1 g and subsequently placed in an incubator at 37.5 °C with 60% humidity to allow embryonic development and then frozen at minus 20 °C. Since some incubation takes place already before clutch completion, the eggs were incubated differentially according to their laying position to approximately equalize the total incubation time (60 h in case of the first-laid egg, 72 h for the second-laid egg and 84 h in case of the last-laid egg). This also controlled for a potential effect of incubation on testosterone (Elf & Fivizanni, 2002; Eising *et al*., 2003), IgG (Kowalczyk *et al*., 1985) and antioxidant levels. Dummy eggs were used to maintain female incubation behaviour so that we could capture the females after laying of the third egg (within 3 days of egg removal) to obtain body measurements ($n = 7$).

Egg analyses

The collected eggs were defrosted and the yolk and embryo separated. A small tissue sample of the embryo was used for Chelex® resin-based DNA extraction (Walsh *et al*., 1991). Two microlitres of the resulting DNA solution was used in a polymerase chain reaction to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (for details see Griffiths *et al*., 1998). The reliability of this method has been established in earlier studies on this species (e.g. Müller *et al*., 2003).

The yolks were homogenized for the analysis of hormones, IgG and antioxidants. In all cases, all eggs of a clutch were analysed in the same assay.

Hormone analysis

For hormone analysis, about half of the homogenized yolk was diluted with 1 mL water per gram of yolk and about 150 mg of this emulsion was used for hormone analysis. We followed a standard procedure according to Schwabl (1993) with a slight modification. Briefly, samples were extracted twice with 4 mL petroleum ether/diethylether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently, the hormones were separated on diatomaceous earth chromatography columns. Testosterone concentrations were measured in double competitive-binding radioimmunoassays (RIA) with tritiated hormone (Testosterone: NET 553; Androstenedione: NET 469, Perkin-Elmer Nederland BV, Groningen, NL) and hormone-specific IgG (Endocrine Science, Calabasas Hills, CA, USA). The average recovery was 49.4%, the intra-assay variation was 4.2%.

Immunoglobulin assay

Immunoglobulin concentrations in plasma and yolk homogenate were determined after 10-fold dilution (w/w) with an anionic detergent buffer (0.33% sodium dodecyl sulphate in 0.5 M Tris–HCl pH 6.8 and 10% glycerol). This is the standard sample buffer for protein separation using polyacrylamide gel electrophoresis (Harlow & Lane, 1999), which we used to resolve the spectrum of egg-yolk proteins. Gull IgG was identified on the basis of molecular weight of the denatured molecule and the molecular weight of the subunits produced under reducing conditions as outlined in Apanius *et al.* (1983). IgG concentration was measured with a quantitative Coomassie G-250 staining protocol (Neuhoff *et al*., 1988) using a standard curve based on purified chicken IgG (Sigma I4881) and expressed in mg mL^{-1} of plasma and mg mg^{-1} of yolk. Randomly chosen plasma ($n = 23$ individual females) and yolk ($n = 36$ eggs) samples were analysed twice and the repeatability of the method (intraclass correlation coefficient) was estimated to be 0.977 ($F_{22,23} = 45.18$,

$P < 0.0001$) and 0.683 ($F_{35,36} = 2.46$, $P < 0.0032$) respectively. In one clutch only two of the three eggs could be measured successfully, hence we excluded the complete clutch from the analysis of IgG.

Carotenoid and vitamin E assays

An aliquot of yolk (~200 mg) was mixed with 0.5 mL 5% sodium chloride by vortexing. Next, 1 mL ethanol was added to the mixture and homogenized for 20 s, then 2 mL hexane was added and the mixture was homogenized for a further 20 s. After centrifugation the lipophilic hexane phase was collected. Extraction using hexane was performed once more. The combined hexane phase was evaporated to dryness under a stream of nitrogen gas and then redissolved in 0.3 mL dichloromethane–methanol (1 : 1) ready for high-performance liquid chromatography (HPLC). For analysis of total carotenoids, samples (10 μL) were injected into an HPLC system fitted with a Spherisorb type S5NH2, 5 μm C_{18} reverse-phase column (25 cm × 4.6 mm) (Phase Separations, Clwyd, UK) and a mobile phase of methanol-distilled water (97 : 3) at a flow rate of 1.5 mL min^{-1}. Carotenoids were identified as a single peak at 445 nm and the concentration determined using lutein (Sigma-Aldrich, Poole, UK) in methanol as a standard. For analysis of vitamin E (α- and γ-tocopherol), samples (10 μL) were injected into an HPLC system fitted with a Spherisorb type S30DS2, 3 μm C_{18} reverse-phase column (15 cm × 4.6 mm) (Phase Separations), and a mobile phase of methanol-distilled water (97 : 3) at a flow rate of 1.05 mL min^{-1}. Fluorescence detection of vitamin E involved excitation and emission wavelengths of 295 and 330 nm, respectively, and the concentration determined in relation to solutions of α-tocopherol and γ-tocopherol (Sigma-Aldrich) in methanol. Tocol was used as an internal standard. Concentrations are given in μg g^{-1} of yolk. Nineteen complete clutches were analysed.

Statistical analyses

None of the data sets deviate significantly from a normal distribution. In the first approach, we analysed the within-clutch allocation pattern using hierarchical linear models in the MLwiN program 1.10 (Rasbash *et al.*, 2000). This method allows analyses of variance and covariance taking into account the nested relationship of different eggs in a nest and controls for multiple (independent) variables. Significance was tested using the increase in deviance (Δdeviance), when a factor was removed from the model, which follows a chi-squared distribution (Wald statistic). Position in the laying sequence, offspring sex and their interaction were included as categorical predictors. Since we were interested in within-nest effects of offspring sex independently from sex differences at the total clutch level, the clutch sex ratio was included in the model too (Snijders & Bosker, 1999).

In a second approach, we investigated the potential relationships between levels of testosterone IgG, antioxidants and egg mass in individual eggs. To this end we calculated for each of these components the residuals over the laying sequence in the MLwiN program and subsequently applied parametric correlation analyses (Pearson, SPSS, Chicago, IL, USA). In a third approach, we correlated total clutch levels among the different yolk components.

Finally, we correlated the change over the laying order among the different egg components. The increase in testosterone levels was calculated as the concentration of last egg minus that of the first egg. The decrease in other components was calculated as the concentration of the first egg minus that of the last egg. Since the biological effect of a certain absolute change is very likely to be stronger in case of low levels than of high levels of that compound in the clutch, we calculated relative changes over the laying order by dividing the changes over the laying sequence by the concentration of the first egg (see Müller *et al.*, 2004a).

Levels of vitamin E and carotenoids very strongly correlated and showed similar trends in all analyses. Therefore, and because of their similar antioxidant function, we used the sum of both concentrations as a measure of total antioxidant concentrations in most analyses.

The relation between body mass of mother gulls and clutch levels and relative changes over the laying sequence of the four egg components ($n = 7$ except for antioxidant levels where $n = 6$) was analysed by means of Pearson correlations.

Results

Variation of the separate components within clutches

Egg mass changed slightly with the laying sequence (Fig. 1a; laying sequence: $\Delta\text{deviance}_1 = 12.08$, $P = 0.002$), independent of sex, or sex in interaction with laying sequence (Table 1; sex: $\Delta\text{deviance}_1 = 0.79$, $P = 0.37$; sex × laying sequence: $\Delta\text{deviance}_2 = 0.30$, $P = 0.86$). *Post hoc* tests revealed that the second-laid egg was significantly heavier than the other eggs (*post hoc*, first vs. second: $\Delta\text{deviance}_1 = 4.59$, $P = 0.03$; second vs. third: $\Delta\text{deviance}_1 = 11.76$, $P < 0.001$; first vs. third: $\Delta\text{deviance}_1 = 2.42$, $P = 0.11$).

Yolk IgG concentrations decreased across the laying sequence (Fig. 1b; laying sequence: $\Delta\text{deviance}_2 = 11.90$, $P < 0.005$). There was neither a significant difference in yolk IgG concentrations between male and female eggs (Table 1; sex: $\Delta\text{deviance}_1 = 0.04$, $P = 0.84$) nor an effect of sex in interaction with laying sequence (sex × laying sequence: $\Delta\text{deviance}_2 = 2.88$, $P = 0.24$).

Yolk antioxidant concentrations consistently decreased from first to last laid egg (Fig. 1c; laying sequence: $\Delta\text{deviance}_2 = 13.20$, $P < 0.001$). Again, neither sex nor the interaction between sex and laying sequence

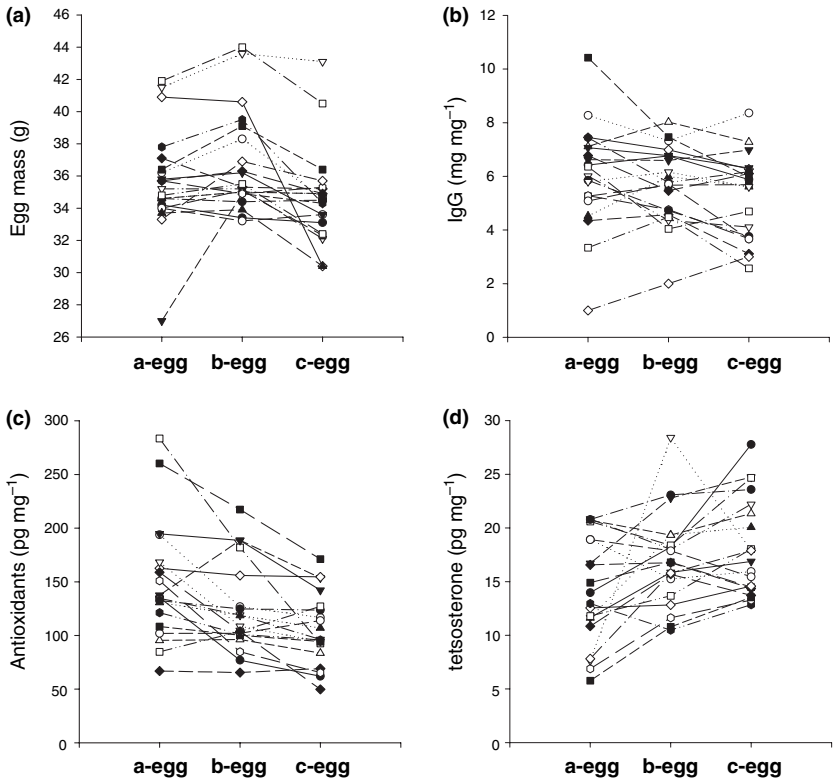

Fig. 1 (a) Mean egg mass (±SE) and mean concentrations (±SE) of (b) IgG, (c) testosterone and (d) antioxidants in relation to the position of the egg in the laying sequence (a-egg, first-laid egg; b-egg, second-laid egg and c-egg, third-laid egg).

Table 1 Mean egg mass (±SE) and mean concentration (±SE) of all egg components analysed in this study, separated for laying position of the egg and sex of the embryo.

	a-egg				b-egg				c-egg			
	n	Male	*n*	Female	*n*	Male	*n*	Female	*n*	Male	*n*	Female
Egg mass	9	36.54 ± 1.55	11	35.05 ± 0.41	9	36.62 ± 0.75	11	36.88 ± 1.15	9	34.17 ± 0.63	11	34.91 ± 1.12
Vit E α	9	109.69 ± 16.19	10	105.37 ± 13.23	8	74.10 ± 6.82	11	99.57 ± 11.31	8	71.46 ± 11.23	11	80.65 ± 5.92
Vit E γ	9	3.85 ± 1.04	10	4.76 ± 1.25	8	2.57 ± 0.60	11	4.57 ± 0.83	8	2.11 ± 0.76	11	2.58 ± 0.38
Carotenoids	9	37.42 ± 4.71	10	36.14 ± 2.60	8	29.21 ± 2.62	11	33.71 ± 3.27	8	22.94 ± 2.83	11	29.49 ± 2.94
Total AOX	9	150.97 ± 20.97	10	146.27 ± 15.99	8	105.88 ± 9.52	11	137.85 ± 14.24	8	96.51 ± 13.99	11	112.72 ± 8.63
IgG	8	6.36 ± 0.64	11	6.23 ± 0.45	8	6.03 ± 0.39	11	5.76 ± 0.38	9	5.80 ± 0.43	10	4.96 ± 0.53
Testosterone	9	14.28 ± 1.50	11	13.99 ± 1.68	9	15.88 ± 1.75	11	18.0 ± 1.05	9	17.33 ± 1.56	11	18.84 ± 1.34

(Vit E α, vitamin E α-tocopherol; Vit E γ, vitamin E γ-tocopherol; AOX: combined antioxidants; IgG: Immunoglobulin G; a-egg, first-laid egg; b-egg; second-laid egg; c-egg, last-laid egg.

contributed significantly to the explained variation in antioxidant titres (Table 1; sex: $\Delta\text{deviance}_1 = 0.45$, $P = 0.50$; sex × laying sequence: $\Delta\text{deviance}_2 = 0.69$, $P = 0.71$). The significant decrease in laying order was present in all antioxidant components measured (total carotenoids: $\Delta\text{deviance}_2 = 18.68$, $P < 0.001$; vitamin E α-tocopherol: $\Delta\text{deviance}_2 = 14.61$, $P < 0.001$; vitamin E γ-tocopherol: $\Delta\text{deviance}_2 = 18.22$, $P < 0.001$). The separated values for all three components are shown in Table 1.

Consistent with earlier studies in black-headed gulls, yolk testosterone concentrations increased across the laying sequence (Fig. 1d; laying sequence: $\Delta\text{deviance}_2 = 13.00$, $P = 0.002$). There were no significant associations between sex of the egg or sex in interaction with laying sequence and yolk testosterone concentration (Table 1; sex: $\Delta\text{deviance}_1 = 1.66$, $P = 0.20$; sex × laying sequence: $\Delta\text{deviance}_2 = 0.19$, $P = 0.91$).

Covariation among components

There was no statistically significant correlation among the residuals over the laying sequence of any of the four egg components presented in Fig. 1 (Table 2, first two columns). This was also the case for variation at the level of the clutch (Table 2, columns 3 and 4). The relative

Table 2 Pearson correlation coefficients and related *P*-values for the correlations among the four egg components in the three levels of analyses.

	Residuals		Clutch level		Relative change	
	Pearson	*P*	Pearson	*P*	Pearson	*P*
Egg mass						
IgG	0.05	0.71	−0.22	0.36	0.16	0.51
AOX	0.21	0.12	0.35	0.14	0.30	0.22
Test	0.18	0.18	0.04	0.88	−0.11	0.65
IgG						
AOX	−0.01	0.97	−0.18	0.47	−0.15	0.54
Test	0.02	0.90	−0.12	0.63	−0.04	0.87
AOX						
Test	−0.12	0.36	0.13	0.61	0.47	0.04

AOX, combined antioxidants; see text for further details.

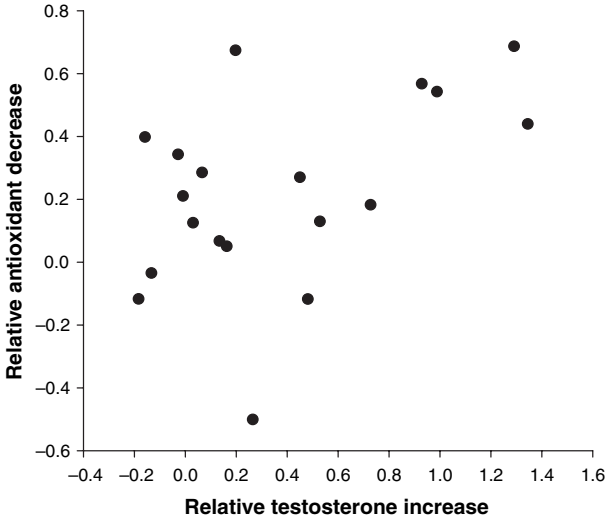

Fig. 2 Relative decrease in antioxidant concentration between the first-laid and last-laid egg against the relative increase in testosterone concentration between the first-laid (a-egg) and the last-laid egg (c-egg).

increase in testosterone concentrations was larger when the decrease in antioxidant concentrations was also larger (Table 2 last two columns, Fig. 2).

Maternal body mass and egg components

Female body mass was not correlated with clutch mass (Pearson correlation coefficient $r = 0.09$, $n = 7$, $P = 0.85$), clutch yolk IgG concentrations ($r = 0.66$, $n = 7$, $P = 0.11$), clutch yolk antioxidant concentrations ($r = 0.36$, $n = 6$, $P = 0.49$) or clutch yolk testosterone levels ($r = -0.25$, $n = 7$, $P = 0.59$). Female body mass was negatively correlated with the relative decrease in antioxidant concentration ($r = -0.94$, $n = 6$, $P = 0.006$) and was on the verge of a significant negative correlation with the increase in testosterone ($r = -0.75$, $n = 7$, $P = 0.05$). Female body mass was unrelated to the relative decrease in egg mass ($r = -0.03$, $n = 7$, $P = 0.94$) or IgG concentrations ($r = 0.42$, $n = 7$, $P = 0.34$) across the laying sequence.

Discussion

To increase our understanding of the adaptive function and evolution of maternal effects, we studied maternal deposition patterns of four substances relevant to immune defence in black-headed gull eggs. We were interested to what extent these patterns relate to hatching asynchrony and sex of the embryo, and whether they show mutual adjustment. We hypothesized that evolution has favoured avian mothers to compensate for the possible immunosuppressive effects of elevated levels of testosterone, required for mitigating the negative consequences of hatching asynchrony for the last hatchlings, with higher levels of antioxidants and IgG; especially so in later-laid eggs that contain relatively high levels of androgens, and eggs containing male embryos, the more vulnerable sex to early post-hatching mortality. The results do not support these predictions and reveal some interesting patterns that are contrary to our hypotheses.

Changes within the laying sequence

Maternally derived antioxidant and immunoglobulin (IgG) concentrations in the yolk decreased within the laying sequence (Fig. 1b,c), as has been shown previously for the closely related lesser black-backed gull (Royle *et al.*, 2001; Blount *et al.*, 2002, 2004). The most parsimonious explanation for this pattern is maternal depletion.

Females must obtain antioxidants from the diet (Brush, 1990). Depending on their availability, the foraging ability of the mother, and the requirements for the mother's own antioxidant activity, mother gulls may face limitations in the amount of antioxidants they can transfer to their offspring. This is supported by the fact that under natural feeding conditions, the yolk of lesser black-backed gulls is not maximally provided with antioxidants. Supplemental feeding with a carotenoid-enriched diet resulted in an almost two-fold increase of the yolk carotenoid concentrations (Blount *et al.*, 2002). Interestingly, yolk carotenoid levels still declined over the laying sequence despite the additional supplement that was available until clutch completion. This may reflect competition among oocytes, with higher rates of yolking of the oocytes that ovulate first (Meathrel, 1991).

Like with the antioxidants, the transfer of maternal IgG to the yolk probably represents a cost to the mother, in particular during the period of rapid growth of the oocyte (Kowalczyk *et al.*, 1985). However, the metabolic cost of

immunoglobulin transfer to eggs is uncertain. It has been estimated that the fraction of the IgG pool that is transferred into oocytes is 10–20% for chicken (Kowalczyk *et al*., 1985) and 1% for turkey (Dohms *et al*., 1978). Furthermore, in barn swallows (*H. rustica*), specific antibodies in eggs from immunized females did not vary across the laying sequence (Saino *et al*., 2003). It is, therefore, difficult to argue that declining IgG levels within the laying sequence are due to simple physiological depletion of systemic IgG production (Lochmiller & Deerenberg, 2000). However, differences in physiology related to egg production between species should be considered here. In gull species egg production is relatively costly (Monaghan *et al*., 1998). In addition, immunoglobulin synthesis during egg production is under hormonal control (Barua *et al*., 1998), and the early onset of incubation in gulls may constrain the deposition of IgGs in last-laid eggs.

The decline in egg mass over the laying sequence can be explained in terms of macronutrient depletion, i.e. later oocytes are disadvantaged during the process of yolk formation as less maternal nutrient reserves are available. The rate of the decrease in egg mass across the laying sequence may reflect the mother's current foraging intake of nutrients. Thus later oocytes have a disadvantage as the process of yolk formation is not independent for each separate oocyte and maternal reserves deteriorate during laying. The steepness of the decrease in egg quality over the laying sequence may reflect the mother's capacity for compensatory antioxidant deposition and potentially IgG synthesis. Indeed, clutches produced by more brightly coloured females, indicating higher body carotenoid levels, showed a smaller decrease in carotenoid levels between second and third eggs (Blount *et al*., 2002). To counteract this decline, females may prolong the laying interval between their eggs to provide them sufficiently with resources. This may entail the cost of increased hatching asynchrony since incubation starts already early after laying of the first egg possibly as a means to suppress egg predation and a decline in their viability (Webb, 1987; Brouwer & Spaans, 1994; Müller *et al*., 2004a).

Gulls produce large eggs and we suggest that the decline of egg quality over the laying sequence is the result of a shift in the optimum for the mother. Since black-headed gulls rarely rear the full brood, the third egg is probably an insurance for loss of the first or second egg (Graves *et al*., 1984; Stoleson & Beissinger, 1995; Forbes *et al*., 1997). In case of no loss, chicks of third eggs usually die in the first week after hatching, as a consequence of hatching asynchrony. Therefore, chicks of last-laid eggs have a much lower survival probability than those of earlier laid eggs (Müller *et al*., 2003), and the lower quality of the last less valuable egg may, therefore, reflect an adaptive maternal strategy in the light of the value of the antioxidants and IgGs for herself. The low quality of the last-laid egg of a clutch explains why its chick has a lower survival even when corrected for hatching asynchrony and egg weight (Parsons, 1975).

Yolk testosterone concentrations significantly increased with laying order, as has been shown in earlier studies on gulls, including the black-headed gull (Fig. 1d; Eising *et al*., 2001; Royle *et al*., 2001; Groothuis & Schwabl, 2002; Verboven *et al*., 2003). In contrast to maternal deposition of antioxidants and macronutrients, androgen deposition is likely to be not costly for the female. Production of steroids is not costly in itself. In case deposition of androgens in the egg requires elevated circulating levels of testosterone in the female, exposure to this hormone might be costly to the mother. However, evidence for such passive transfer is ambiguous (for a review see Groothuis *et al*., 2005b) and enhanced testosterone deposition would require elevated maternal levels of the hormone for only a relatively short time span. The finding that females of low body condition allocated more androgens to the yolk compared to females in good condition (Verboven *et al*., 2003) suggests a compensatory function of these hormones (Groothuis & Schwabl, 2002) without an important cost for the female.

Sex allocation

We expected that male embryos, because of their greater sensitivity to egg quality (Nager *et al*., 1999), would receive greater maternal investment than female embryos. This expectation has been supported by several (e.g. steroids: Petrie *et al*., 2001; Müller *et al*., 2002; egg size: Cordero *et al*., 2000, 2001; IgG: Saino *et al*., 2003) but not all studies (steroids: Schwabl, 1993; Verboven *et al*., 2003, carotenoids: Saino *et al*., 2003). We did not find any indication for sex-specific allocation in this study.

Mutual adjustment

As a consequence of the different deposition patterns across the laying sequence, an indirect positive association between yolk concentrations of immunoglobulins and antioxidants, and a negative association between these two and yolk concentrations of testosterone was found. However, levels of antioxidants and antibodies were not correlated at the level of the individual egg, nor at the level of the clutch, nor in terms of within-clutch variation. This may be due to the fact that antioxidants are markers for diet quality and foraging ability while IgG levels integrate exposure to infectious agents. Such covariation would have been adaptive, as antioxidants protect the maternally derived IgG against catabolism *in vivo* (Haq *et al*., 1996). Our results indicate that mothers cannot interactively allocate these yolk compounds. In contrast there was a direct positive association between the rate of decrease in antioxidants and the rate of increase of the testosterone concentrations over the

laying order. This will likely handicap the immune function of the last-hatched chick even further. Such a steep decrease of yolk antioxidants over the laying order may stem from a limited maternal antioxidant availability, which is supported by our finding that such mothers have a relatively low body mass. Such antioxidant limitation could be amplified in mothers that adaptively increase testosterone synthesis because the anabolic effects of testosterone may promote oxidative stress (von Schantz *et al.*, 1999). The elevated levels of immunosuppressive testosterone and low levels of carotenoids in last-laid eggs have been interpreted as a means of adaptive brood reduction in case of low food availability, while under food conditions that are sufficient for a proper development of immune function enhanced levels of maternal testosterone might help to overcome the disadvantage in sibling competition of the last-hatched chick leading to enhanced survival (Royle *et al.*, 2001). This elegant hypothesis does not take into account the high cost of egg production in gulls (Monaghan & Nager, 1997; Monaghan *et al.*, 1998) or the importance of the third egg as an insurance against failure of earlier eggs (see above). We suggest that the decrease in antioxidants with laying order is a constraint of the laying female, whereas enhanced testosterone allocation to last-laid eggs serves as a mechanism to enhance competitiveness (Schwabl, 1993; Eising & Groothuis, 2003), which is especially relevant in eggs of poor quality. In addition we would like to suggest that the possible immunomodulatory effects of yolk testosterone may be interpreted as an adaptive maternal effect. For a growing chick the costs of raising an immune response should be traded against the resulting reduction in growth and the potential loss of a size advantage in respect of sibling rivalry (Brommer, 2003; Soler *et al.*, 2003). As a consequence of the allocation of high amounts of energy to growth last-hatched chicks have a higher vulnerability to infectious diseases, but without this biased allocation they would probably have died anyway. Therefore, under the constraints that mother gulls face both in their ability to maintain egg quality over the laying sequence and in the necessity of early incubation, leading to hatching asynchrony, our results suggest that the allocation of testosterone reflects an adaptive maternal strategy.

In conclusion, we have documented multiple pathways for maternal effects on offspring phenotype. We suggest that evolution has not strongly selected for mechanisms that allow the mother to adjust her deposition of antioxidants and IgGs into eggs to compensate for possible immunomodulatory effects of maternal testosterone. This may in part be explained by maternal constraints. Indeed our results suggest that evolution has favoured increased deposition of testosterone, possibly the less costly pathway for the mother, as a compensation for low egg quality reflected in low levels of antioxidants.

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References

Apanius, V. 1998. Ontogeny of immune function. In: *Avian Growth and Development – Evolution Within the Altricial–Precocial* (J.M. Starck & R.E. Ricklefs, eds), pp. 203–222. Oxford University Press, Oxford.

Apanius, V., Temple, S.A. & Bale, M. 1983. Serum-proteins of wild turkey vultures (*Cathartes aura*). *Comp. Biochem. Physiol. B* **76**: 907–913.

Barua, A., Yoshimura, Y. & Tamura, T. 1998. Effects of ageing and oestrogens on the localization of immunoglobulin-containing cells in the chicken ovary. *J Reprod. Fertil.* **114**: 11–16.

Blount, J.D., Surai, P.F., Nager, R.G., Houston, D.C., Møller, A.P., Trewby, M.L. & Kennedy, M. 2002. Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proc. R. Soc. Lond. B* **269**: 29–36.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. & Surai, P.F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* **300**: 125–127.

Blount, J.D., Houston, D.C., Surai, P.F. & Møller, A.P. 2004. Egg laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. *Proc. R. Soc. Lond. B* **3** (Suppl): 79–81.

Brommer, J.E. 2003. Immunocompetence and its costs during development: an experimental study in blue tit nestlings. *Proc. R. Soc. Lond. B* **271** (Suppl. 3): 110–113.

Brouwer, A. & Spaans, A.L. 1994. Egg predation in the Herring Gull *Larus argentatus*: why does it vary so much between nests? *Ardea* **82**: 223–230.

Brown, C.R. & Brown, M.B. 1986. Ectoparasitism as a cost of coloniality in cliff swallows (*Hirundo pyrrhonota*). *Ecology* **67**: 1206–1218.

Brush, A.H. 1990. Metabolism of carotenoid pigments in birds. *FASEB J.* **4**: 2969–2977.

Buechler, K., Fitze, P.S., Gottstein, B., Jacott, A. & Richner, H. 2002. Parasite-induced maternal response in a natural bird population. *J. Anim. Ecol.* **71**: 247–252.

Christians, J.K. 2002. Avian egg size: variation within species and inflexibility within individuals. *Biol. Rev.* **77**: 1–26.

Cordero, P.J., Griffith, S.C., Aparicio, J.M. & Parkin, D.T. 2000. Sexual dimorphism in house sparrow eggs. *Behav. Ecol. Sociobiol.* **48**: 353–357.

Cordero, P.J., Viñuela, J., Aparicio, J.M. & Veiga, J.P. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *J. Evol. Biol.* **14**: 829–834.

Cramp, S. & Simmons, K.E.L. (eds) 1983. *Handbook of the Birds of Europe, the Middle East and North Africa: the Birds of the Western Paleartic. Volume III: Waders to Gulls*. Oxford University Press, Oxford.

Dohms, J.E., Saif, Y.M. & Bacon, W.L. 1978. Metabolism and passive transfer of immunoglobulins in the turkey hen. *Am. J. Vet. Res.* **39**: 1472–1481.

Duffy, D.L., Bentley, G.E., Drazen, D.L. & Ball, G.F. 2000. Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. *Behav. Ecol.* **11**: 654–662.

Eising, C.M. & Groothuis, T.G.G. 2003. Yolk androgens and begging behaviour in black-headed gull chicks: an experimental field study. *Anim. Behav.* **66**: 1027–1034.

Eising, C.M., Eikenaar, C., Schwabl, H. & Groothuis, T.G.G. 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond. B* **268**: 839–846.

Eising, C., Müller, W., Dijkstra, C. & Groothuis, T.G.G. 2003. Maternal androgens in egg yolks: relation with sex, incubation time and embryonic growth. *Gen. Comp. Endocrinol.* **132**: 241–247.

Elf, P.K. & Fivizanni, A.J. 2002. Changes in sex steroid levels in yolks of the Leghorn chicken, *Gallus domesticus*, during embryonic development. *J. Exp. Zool.* **293**: 594–600.

Forbes, L.S., Thornton, S., Glassey, B., Forbes, M. & Buckley, N. 1997. Why parent birds play favourites. *Nature* **390**: 351–352.

Gasparini, J., McCoy, K.D., Tveraa, T. & Boulinier, T. 2001. Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial seabird the kittiwake *Rissa tridactyla*. *Proc. R. Soc. Lond. B.* **268**: 647–650.

Graves, J., Whiten, A. & Henzi, P. 1984. Why does the herring gull lay three eggs? *Anim. Behav.* **32**: 798–805.

Griffiths, R., Double, M.C., Orr, K. & Dawson, R.J.G. 1998. A DNA test to sex most birds. *Mol. Ecol.* **7**: 1071–1075.

Grindstaff, J.L., Brodie, E.D. III & Ketterson, E.D. 2003. Immune function across generations: integrating mechanism and evolutionary process in maternal IgG transmission. *Proc. R. Soc. Lond. B.* **270**: 2309–2319.

Groothuis, T.G.G. & Schwabl, H. 2002. The influence of laying sequence and habitat characteristics on maternal yolk hormone levels. *Funct. Ecol.* **16**: 281–289.

Groothuis, T.G.G., Eising, C.M., Dijkstra, C. & Müller, W. 2005a. Balancing between costs and benefits of maternal hormone deposition in avian eggs. *Biol. Lett.* **1**: 78–81.

Groothuis, T.G.G., Müller, W., von Engelhardt, N., Carere, C. & Eising, C. 2005b. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* **29**: 329–352.

Haq, A., Bailey, C.A. & Chinnah, A. 1996. Effect of β-carotene, canthaxanthin, lutein and vitamin E on neonatal immunity of chicks when supplemented in broiler diets. *Poult. Sci.* **75**: 1092–1097.

Harlow, E. & Lane, D. 1999. *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hasselquist, D., Marsh, J.A., Sherman, P.W. & Wingfield, J.C. 1999. Is avian humoral immunocompetence suppressed by testosterone? *Behav. Ecol. Sociobiol.* **45**: 167–175.

Hirota, Y., Suzuki, T., Chazono, Y. & Bito, Y. 1976. Humoral immune response characteristics of testosterone-propionate-treated chickens. *Immunology* **30**: 341–348.

Kariyawasam, S., Wilikie, B.N. & Gyles, C.L. 2004. Resistance of broiler chickens to *Escherichia coli* respiratory tract infection induced by passively transferred egg–yolk antibodies. *Vet. Microbiol.* **98**: 273–284.

Ketterson, E.D. & Nolan, V. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *Am. Nat.* **154**: 4–25.

Kowalczyk, K., Daiss, J., Halpern, J. & Roth, T.F. 1985. Quantitation of maternal–fetal IgG transport in the chicken. *Immunology* **54**: 755–762.

Lipar, J.L. & Ketterson, E.D. 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Aegelius phoeniceus*. *Proc. R. Soc. Lond. B* **267**: 2005–2010.

Lochmiller, R.L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**: 87–98.

Loye, J.E. & Zuk, M. 1991. *Bird–Parasite Interactions*. Oxford University Press, Oxford.

Marsh, J.A. 1992. Neuroendocrine-immune interactions in the avian species – a review. *Poult. Sci. Rev.* **4**: 129–167.

McGraw, K.J. & Ardia, D.R. 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *Am. Nat.* **162**: 704–712.

Meathrel, C.E. 1991. Variation in eggs and the period of rapid yolk deposition of the silver gull *Larus Novaehollandiae* during a protracted laying season. *J. Zool.* **223**: 501–508.

Monaghan, P. & Nager, R.G. 1997. Why don't lay birds more eggs? *TREE* **12**: 270–274.

Monaghan, P., Nager, R.G. & Houston, D.C. 1998. The price of eggs: increased investment in egg production reduces the offspring rearing capacity of parents. *Proc. R. Soc. Lond. B* **265**: 1731–1735.

Mousseau, T.A. & Fox, C.W. (eds) 1998. *Maternal Effects*. Oxford University Press, New York.

Müller, W., Eising, C., Dijkstra, C. & Groothuis, T.G.G. 2002. Sex differences in yolk hormones depend on maternal social status in Leghorn chickens (*Gallus gallus domesticus*). *Proc. R. Soc. Lond. B* **269**: 2249–2256.

Müller, W., Dijkstra, C. & Groothuis, T.G.G. 2003. Inter-sexual differences in T-cell-mediated immunity of black-headed gull chicks (*Larus ridibundus*) depend on the hatching order. *Behav. Ecol. Sociobiol.* **55**: 80–86.

Müller, W., Eising, C.M., Dijkstra, C. & Groothuis, T.G.G. 2004a. Within-clutch patterns of yolk testosterone vary with the onset of incubation in black-headed gulls. *Behav. Ecol.* **15**: 893–897.

Müller, W., Dijkstra, C., Groothuis, T.G.G., Siitari, H. & Alatalo, A.V. 2004b. Maternal antibody transmission and breeding densities in the black-headed gull (*Larus ridibundus*). *Funct. Ecol.* **18**: 719–724.

Müller, W., Groothuis, T.G.G., Dijkstra, C., Kasprzik, A., Alatalo, R.V. & Siitari, H. 2005. Prenatal androgen exposure modulates cellular and humoral immune function of black-headed gull chicks. *Proc. R. Soc. Lond. B.*, in press.

Nager, R.G., Monaghan, P., Griffiths, R., Houston, D.C. & Dawson, R. 1999. Experimental evidence that off spring sex ratio varies with maternal condition. *Proc. Natl Acad. Sci. USA* **96**: 570–573.

Nager, R.G., Monaghan, P., Houston, D.C. & Genovart, M. 2000. Parental condition, brood sex ratio and differential young survival: an experimental study in gulls (*Larus fuscus*). *Behav. Ecol. Sociobiol.* **48**: 452–457.

Neuhoff, V., Arold, N., Taube, D. & Ehrhardt, W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**: 255–262.

Parsons, J. 1975. Relationship between egg size and post-hatching chick mortality in the herring gull (*Larus argentatus*). *Nature* **228**: 1221–1222.

Peters, A. 2000. Testosterone treatment is immunosuppressive in superb fairy-wrens, yet free-living males with high testosterone are more immunocompetent. *Proc. R. Soc. Lond. B* **267**: 883–889.

Petrie, M., Schwabl, H., Brande-Lavridsen, N. & Burke, T. 2001. Sex differences in avian yolk hormone levels. *Nature* **412**: 498.

Rasbash, J., Browne, W., Healy, M., Cameron, B. & Charlton, C. 2000. *Multilevel Models Project*. University of London, London.

Ros, A.F.H., Groothuis, T.G.G. & Apanius, V. 1997. The relationship between gonadal steroids, immunocompetence, body mass, and behavior in young black-headed gulls (*Larus ridibundus*). *Am. Nat.* **150**: 201–219.

Royle, N.J., Surai, P.F. & Hartley, I.R. 2001. Maternal derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behav. Ecol.* **12**: 381–385.

Saino, N., Romano, M., Ferrari, R.P., Martinelli, R. & Møller, A.P. 2003. Maternal IgG but not carotenoids in barn swallow eggs covary with embryo sex. *J. Evol. Biol.* **16**: 516–522.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B* **266**: 1–12.

Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl Acad. Sci. USA* **90**: 11446–11450.

Schwabl, H. 1996. Maternal testosterone in the egg enhances postnatal growth. *Comp. Biochem. Physiol.* **114**: 271–276.

Sheldon, B.C. & Verhulst, S. 1996. Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. *TREE* **11**: 317–321.

Snijders, T.A.B. & Bosker, R.J. 1999. *Multilevel Analysis; An Introduction to Basic and Advanced Multilevel Modeling*. Sage Publications, London.

Sockman, K.W. & Schwabl, H. 2000. Yolk androgens reduce offspring survival. *Proc. R. Soc. Lond. B.* **267**: 1451–1456.

Soler, J.J., de Neve, L., Perez, T., Soler, M. & Sorci, G. 2003. Trade-off between immunocompetence and growth in magpies: an experimental study. *Proc. R. Soc. Lond. B* **270**: 241–248.

Stoleson, S.H. & Beissinger, S.R. 1995. Hatching asynchrony and the onset of incubation in birds, revisited: when is the critical period? In: *Current Ornithology*, Vol. 12 (D.M. Power, ed.), pp. 191–271. Plenum, New York.

Surai, P.F. 2002. *Natural Antioxidants in Avian Nutrition and Reproduction*. Nottingham University Press, Nottingham, UK.

Surai, P.F., Speake, B.K., Noble, R.C. & Sparks, N.H.C. 1999. Tissue specific antioxidant profiles and susceptibility to lipid peroxidation of the newly hatched chick. *Biol. Trace Elem. Res.* **68**: 63–78.

Tella, J.L. 2002. The evolutionary transition to coloniality promotes higher blood parasitism in birds. *J. Evol. Biol.* **15**: 32–41.

Tschirren, B., Richner, H. & Schwabl, H. 2004. Ectoparasite-modulated deposition of maternal androgens in great tit eggs. *Proc. R. Soc. Lond. B* **271**: 1370–1375.

Verboven, N., Monaghan, P., Evans, D.M., Schwabl, H., Evans, N., Whitelaw, C. & Nager, R.G. 2003. Maternal condition, yolk androgens and offspring performance: a supplemental feeding experiment in the lesser black-backed gull (*Larus fuscus*). *Proc. R. Soc. Lond. B* **270**: 2223–2232.

Walsh, P.S., Metzger, D.A. & Higuchi, R. 1991. Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**: 506–513.

Webb, D.R. 1987. Thermal tolerance of avian embryos: a review. *Condor* **89**: 874–898.

Williams, T.D. 1994. Intraspecific variation in egg size and egg composition in birds – effects on offspring fitness. *Biol. Rev.* **69**: 35–59.

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